Further Characterization of Glycolaldehyde Dehydrogenase Isozymes from *Escherichia coli* B Involvement in Vitamin B₆ Biosynthesis[†]

Hiroshi MORITA,*¹ Yoshiki TANI, Koichi OGATA*²
and Hideaki YAMADA

Department of Agricultural Chemistry, Kyoto University, Kyoto 606, Japan

Received June 2, 1978

Kinetic study of glycolaldehyde dehydrogenase was performed with isozymes of *Escherichia coli* B. The reaction equilibrium of isozyme A was estimated to lie in glycolate formation, while those of isozymes B and C were in glycolaldehyde formation. Isozyme A was released from cells with osmotic shock, while the others were not. Isozymes B and C were found in cytoplasmic fraction. Some reversal mutants derived from WG3 strain (one of vitamin B₆ auxotrophs) acquired ability to produce isozyme C. Based on these results, the non-involvement of isozyme A in vitamin B₆ biosynthesis was discussed.

In the previous papers,^{1~4)} we reported the involvement of glycolaldehyde dehydrogenase in vitamin B₆ biosynthesis in *Escherichia coli* B. The presence of isozymes (A, B and C) of the enzyme was also shown in a wild type of *E. coli* B.³⁾ The activity of isozyme B was activated by pyridoxine and that of isozyme C was inhibited by pyridoxine. No vitamin B₆ compound affected the activity of isozyme A. From these results, isozyme A was not assumed to be involved in the vitamin B₆ biosynthetic pathway. In this paper, we investigated the reaction equilibrium and the subcellular localization of these isozymes to elucidate the mechanism of enzyme participation in vitamin B₆ biosynthesis.

MATERIALS AND METHODS

Chemicals. All chemicals were usual commercial products and used without further purification.

Microorganisms. The organisms used were *Escherichia coli* B WG1 (wild type); WG3 and WG150 (vitamin B₆ auxotrophs which lack *pdxB3* gene); and WG3-r4, WG3-r6 and WG3-r10 (reversal mutants derived from

WG3 strain), which were gifts from W.B. Dempsey.

Enzyme assay. Glycolaldehyde dehydrogenase activity was determined by the method described previously,³⁾ unless otherwise stated. Alkaline phosphatase, fumarase and nitrate reductase activities were assayed by the methods of Omori,⁵⁾ Hill and Bradshaw,⁶⁾ and Taniguchi and Itagaki.⁷⁾ EDTA was removed from enzyme samples by dialysis, when enzyme activity other than glycolaldehyde dehydrogenase was assayed.

Preparation of the isozymes. Each glycolaldehyde dehydrogenase isozyme was prepared by the method described previously.³⁾

Subcellular localization of enzyme. For isolation of enzymes located in periplasmic space, *E. coli* B WG1 was treated by the modified method of Brockman and Heppel.⁸⁾ Namely, the organism was cultivated in 5 liters of glycerol-casamino acid medium for 26 hr at 28°C. The cells were harvested (26.7 g wet weight) and suspended in 133 ml of 33 mM Tris-HCl buffer (pH 7.3). An equal volume of 40% (w/v) sucrose solution in 33 mM Tris-HCl buffer (pH 7.3) was added to the cell suspension. Then, 500 mM of EDTA·2Na (pH 7.0) was added to the cell suspension to a final concentration of 1 mM. The suspension was treated by rotary shaking at 60 rpm at room temperature for 10 min. The plasmolyzed cells were collected by centrifugation at 10,000 × *g* for 12 min. The pellets formed were suspended into 810 ml of ice chilled 0.5 mM MgCl₂. The above suspension was treated with rotary shaking for 10 min in the same manner described above. In this process, enzymes located in the periplasmic space were released by osmotic shock. The shocked cells were harvested by centrifugation at 10,000 × *g* for 15

[†] Studies on Vitamin B₆ Metabolism in Microorganisms. Part XVI. For Part XV, see *J. Nutr. Sci. Vitaminol.*, 24, 229 (1978).

*¹ Present address: Kyoto College of Nutritional Sciences, 18 Tenryujisetogawacho, Saga, Ukyo-ku, Kyoto 615, Japan.

*² Deceased.

TABLE I. APPARENT MICHAELIS CONSTANTS OF GLYCOLALDEHYDE DEHYDROGENASE ISOZYMES

Substrate	Symbol	Apparent Michaelis constants (M)			
		A ^a	B ^a	C ^a	WG3 A
Glycolate	<i>Kb</i>	6.9×10^{-11}	1.0×10^{-2}	4.9×10^{-5}	5.9×10^{-8}
NADPH ₂	<i>Ka</i>	1.2×10^{-4}	9.4×10^{-5}	2.5×10^{-5}	1.5×10^{-4}
Glycolaldehyde	<i>Kp</i>	2.7×10^{-5}	2.7×10^{-2}	8.3×10^{-3}	1.4×10^{-2}
NADP	<i>Kq</i>	6.7×10^{-5}	1.1×10^{-4}	1.3×10^{-4}	1.5×10^{-4}

^a indicate the glycolaldehyde dehydrogenase isozymes from *E. coli* B WG1.

min. The supernatant formed was dialyzed against 0.01 M potassium phosphate buffer (pH 7.0) containing 0.1% EDTA·3Na, 5 mM of 2-mercaptoethanol and 0.5 mM dithiothreitol. The dialyzed solution was referred to as shocked fluid. The shocked cells were disrupted by a Kaijo-Denki sonic oscillator at 19 kHz for 10 min. The debris was removed from the homogenate by centrifugation at 3000 rpm for 10 min. After the removal of the debris, the homogenate was centrifuged at $105,000 \times g$ for 6 hr. The supernatant formed was referred to as cytoplasmic fraction. The precipitate was suspended in the potassium phosphate buffer containing 1.5 mM of EDTA. The suspension was centrifuged at 6000 rpm for 10 min. The resultant supernatant was referred to as particulate wash. Each subcellular fraction prepared was applied on DEAE-cellulose column for separation of isozymes.

RESULTS

Kinetic study

For reaction equilibrium of isozymes, we determined the apparent Michaelis constants for four substrates (glycolate, NADPH₂, glycolaldehyde and NADP) and the inhibition constants of glycolaldehyde and NADP using product inhibition studies.⁹⁾ (Tables I and II) The activities of isozymes assayed with different

concentration of substrates are summarized in Lineweaver-Burk plots,¹⁰⁾ as shown in Fig. 1. The lines crossed each other on one point. This suggests that each reaction of isozymes operates in sequential mechanism. Results of product inhibition studies are illustrated in Fig. 2. For isozymes A and B, glycolaldehyde inhibited non-competitively against glycolate, and NADP was competitive against NADPH₂. Neither glycolaldehyde nor NADP inhibited the reaction of isozyme C. These reaction patterns of isozymes A and B were consistent with ordered bi bi mechanism, as shown in Scheme. The pattern of isozyme C was consistent with a rapid equilibrium random

SCHEME. Mechanism of Enzymatic Reaction of Glycolaldehyde Dehydrogenase Isozymes.

A, B and C indicate the reaction with isozymes A, B and C, respectively.

TABLE II. INHIBITION CONSTANTS AND RATIO OF MAXIMAL VELOCITY OF FORWARD REACTION-TO-REVERSE REACTION

Kip and *Kib* indicate the inhibition constants of glycolaldehyde and glycolate, respectively. V_1/V_2 indicates the ratio of maximal velocity of forward reaction-to-reverse reaction.

Symbol	Isozymes		
	A	B	C
<i>Kip</i>	$2.1 \times 10^{-6} \text{M}$	$4.0 \times 10^{-6} \text{M}$	$8.3 \times 10^{-3} \text{M}$
<i>Kib</i>	$8.5 \times 10^{-6} \text{M}$	$8.5 \times 10^{-7} \text{M}$	$1.3 \times 10^{-4} \text{M}$
V_1/V_2	0.49	9.52	0.55

mechanism. Inhibition constants of glycolaldehyde or glycolate, and the ratio of maximal velocity of forward reaction-to-reverse reaction was calculated, as shown in Table II. In isozyme C, inhibition constants of glycolaldehyde and glycolate were substituted for the apparent

FIG. 1. Effect of Substrate Concentration on Glycolaldehyde Dehydrogenase Isozymes.

A, B and C represent the reaction with isozymes A, B and C, respectively. The reaction was carried out by the method described in the previous paper.²⁾ U, units of the enzyme activity.

FIG. 2. Inhibitory Effect of Glycolaldehyde on Reaction.

Closed circle (●) represents the reaction assayed in the reaction mixture supplemented with 1.1 μM of glycolaldehyde. Open circle (○) represents the reaction without addition of glycolaldehyde. A, B and C indicate the reaction with isozymes A, B and C, respectively. U, units of the enzyme activity.

Michaelis constants of glycolaldehyde or glycolate, respectively.

From these parameters, the equilibrium constants for isozymes A and B were calculated with the following Haldane's equation.⁹⁾

Isozymes A and B: ordered bi bi mechanism

$$K_{eq} = (V_1/V_2)^2 (K_{ip}/K_a)(K_q/K_{ib})$$

K_{eq} : equilibrium constant

The equilibrium constant for isozyme C was calculated with the following equation.⁹⁾

Isozyme C: rapid equilibrium random mechanism

$$K_{eq} = (V_1/V_2)(K_{ip}/K_a)(K_q/K_{ib})$$

The free energy change of each isozyme was calculated with the following equation.

$$\Delta G = -RT \ln K_{eq}$$

ΔG : free energy change, (cal/mol),

R : gas constant, 1.99 cal·°K/mol,

T : reaction temperature, 310°K.

TABLE III. EQUILIBRIUM CONSTANTS AND FREE ENERGY CHANGE

Affair	Symbol	Glycolaldehyde dehydrogenase isozymes		
		A	B	C
Equilibrium constant	K_{eq}	3.3×10^{-3}	502	480
Free energy change	ΔG	+3490	-2150	-3790

As shown in Table III, the value of the free energy change was positive in isozyme A, so the reaction equilibrium lies in the oxidation of glycolaldehyde in spite of low apparent Michaelis constant for glycolate. On the other hand, the reaction equilibria of isozymes B and C lie in the formation of glycolaldehyde because of the negative values of free energy change.

Subcellular localization of enzyme

To investigate the subcellular localization of the isozyme, we applied each subcellular frac-

TABLE IV. SUBCELLULAR LOCALIZATION OF GLYCOLALDEHYDE DEHYDROGENASE ISOZYMES

Preparation	Relative total activities (%)							
	Glycolaldehyde dehydrogenase				Nitrate reductase	Fumarase	NADPH ₂ oxidase	Alkaline phos- phatase
	Before separation ^a	Isozyme						
		A	B	C				
Cell-free extract	100	27.1	7.10	1.86	100	100	100	100
Shocked fluid	1.51	0.44	0	0	0	0	1.54	39.1
Cytoplasm	56.3	16.2	9.57	2.59	0	15.6	80.7	52.3
Particulate wash	3.0	0.49	1.10	1.51	145	95.0	3.36	15.3

^a Total activity assayed before application on DEAE-cellulose column.

tion on DEAE-cellulose column chromatography, according to the method described previously.³⁾ The activities of each isozyme in each subcellular preparation were determined (Table IV). The shocked fluid contained isozyme A but not isozymes B and C. This result suggests that isozyme A is located in the periplasmic space. The amount ratio of isozyme B plus isozyme C to isozyme A was larger in cytoplasm than in cell-free extract. This suggests that isozymes B and C are located in cytoplasm. The presence of isozyme A in other fractions might be caused by contamination of the cell wall component. The incomplete separation of other marker enzymes by this method supports this presumption.

Glycolaldehyde dehydrogenase isozymes in reversal mutants

Several revertants which can synthesize vitamin B₆ *de novo* were derived from *E. coli* B WG3.¹¹⁾ The activity of glycolaldehyde dehydrogenase of these strains is shown in Table V. All cell-free extracts of revertants showed activity even under assay conditions, in which the activity of WG3 could not be detected.¹⁾ Then we prepared isozymes by DEAE-cellulose column chromatography. Every revertants acquired ability to produce isozyme C, besides r4 strain produced isozyme B. One of the vitamin B₆ auxotrophs, WG150 strain, which belongs to *pdxB3* together with WG3 strain, also had isozyme C.

DISCUSSION

We reported that isozyme A utilizes wide

varieties of aldehydes as substrate and that isozyme B or C catalyzes glycolaldehyde specifically.³⁾ Pyridoxal 5'-phosphate supple-

TABLE V. GLYCOLALDEHYDE DEHYDROGENASE ISOZYMES IN *Escherichia coli* B MUTANTS

Strain	Specific activity ^a		Relative total activity (%) ^b		
	Reduc-tion	Oxi-dation	Isozymes		
			A	B	C
WG1	0.45	2.9	75.2	19.7	5.2
WG3	0	0	100	0	0
WG150	0.42	2.3	89.8	0	10.2
WG3-r4	0.81	2.2	75.9	6.7	17.4
WG3-r6	0.80	0	89.7	0	10.3
WG3-r10	0.59	1.8	83.9	0	16.1

^a Specific activity of glycolate reduction or glycolaldehyde oxidation in cell-free extract prepared and assayed by the method described previously¹⁾; viz., the cell-free extract was dialyzed against 0.01 M potassium phosphate buffer (pH 7.1) containing 5.7 mM 2-mercaptoethanol without addition of EDTA. The reactions were assayed in pH 7.1.

^b The reaction was assayed by the NADPH₂ method described previously.²⁾

ment (10 μ M) represses the syntheses of isozymes B and C completely, but not isozyme A. A 100 μ M of pyridoxine in the reaction mixture inhibited the activity of isozyme C and activated isozyme B, but had no pronounced effect on the activity of isozyme A.⁴⁾ One of the vitamin B₆ auxotrophs, WG3, whose requirement of vitamin B₆ is replaceable with glycolaldehyde, lacks isozymes B and C, but still retains isozyme A.³⁾ These data suggest that isozymes B and C are involved in vitamin

TABLE VI. SUMMARY OF CHARACTERIZATION OF GLYCOLALDEHYDE DEHYDROGENASE ISOZYMES

Isozyme	Localization	Substrate specificity	Feedback repression	Feedback inhibition	Inhibition by metal ion	ΔG
A	Periplasm	Broad	No	No	Strong	+
B	Cytoplasm	Narrow	Yes	No	No	—
C	Cytoplasm	Narrow	Yes	Yes	No	—

B₆ biosynthesis and that isozyme A is not associated with pathway. In this report it was established that the equilibrium of isozyme A reaction lay in glycolaldehyde oxidation and that isozyme A was located in the periplasmic space. These data are summarized in Table VI. Thus, the WG3 strain has alternative requirements of vitamin B₆ and glycolaldehyde, even in the presence of isozyme A.

We found that some reversal mutants of WG3 strain acquired ability to produce of isozyme C. One of them acquired both isozymes B and C. This suggest that at least isozyme C is essential for *de novo* synthesis of vitamin B₆. Recently it was also found that some vitamin B₆ auxotrophs belonging to linkage group I, which lack a *pdxB3* locus as WG3 strain, did not always lack glycolaldehyde dehydrogenase activity.¹¹⁾ Using a mutant of such type, WG150, we detected isozymes A and C but not isozyme B. This may give genetic information on enzyme for the first stage of vitamin B₆ biosynthesis.

Acknowledgement. The authors wish to thank

Dr. W. B. Dempsey, University of Texas Health Science Center at Dallas, for his kind supply of *E. coli* B mutants.

REFERENCES

- 1) Y. Tani, H. Morita and K. Ogata, *Agric. Biol. Chem.*, **38**, 2057 (1974).
- 2) Y. Tani, H. Morita and K. Ogata, *ibid.*, **41**, 1749 (1977).
- 3) Y. Tani, H. Morita, H. Nishise and K. Ogata, *ibid.*, **42**, 63 (1978).
- 4) H. Morita, Y. Tani and K. Ogata, *ibid.*, **42**, 69 (1978).
- 5) S. Omori, *Enzymologia*, **4**, 217 (1937).
- 6) R. L. Hill and R. A. Bradshaw, "Methods in Enzymology," Vol. 13, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, N. Y., 1969, p. 91.
- 7) S. Taniguchi and E. Itagaki, *Biochim. Biophys. Acta*, **44**, 263 (1960).
- 8) R. W. Brockman and L. A. Heppel, *Biochemistry*, **7**, 2554 (1968).
- 9) W. W. Cleland, *Biochim. Biophys. Acta*, **67**, 104, 173 and 188 (1963).
- 10) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
- 11) S. Shimizu and W. B. Dempsey, *J. Bacteriol.*, **133**, 1273 (1978).